

Human Immunodeficiency Virus Type 1 Vpr Induces Cell Cycle G₂ Arrest through Srk1/MK2-Mediated Phosphorylation of Cdc25[∇]

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Human immunodeficiency virus type 1 (HIV-1) Vpr induces cell cycle G₂ arrest in fission yeast (*Schizosaccharomyces pombe*) and mammalian cells, suggesting the cellular pathway(s) targeted by Vpr is conserved among eukaryotes. Our previous studies in fission yeast demonstrated that Vpr induces G₂ arrest in part through inhibition of Cdc25, a Cdc2-specific phosphatase that promotes G₂/M transition. The goal of this study was to further elucidate molecular mechanism underlying the inhibitory effect of Vpr on Cdc25. We show here that, similar to the DNA checkpoint controls, expression of *vpr* promotes subcellular relocalization of Cdc25 from nuclear to cytoplasm and thereby prevents activation of Cdc2 by Cdc25. Vpr-induced nuclear exclusion of Cdc25 appears to depend on the serine/threonine phosphorylation of Cdc25 and the presence of Rad24/14-3-3 protein, since amino acid substitutions of the nine possible phosphorylation sites of Cdc25 with Ala (9A) or deletion of the *rad24* gene abolished nuclear exclusion induced by Vpr. Interestingly, Vpr is still able to promote Cdc25 nuclear export in mutants defective in the checkpoints (*rad3* and *chk1/cds1*), the kinases that are normally required for Cdc25 phosphorylation and nuclear exclusion of Cdc25, suggesting that others kinase(s) might modulate phosphorylation of Cdc25 for the Vpr-induced G₂ arrest. We report here that this kinase is Srk1. Deletion of the *srk1* gene blocks the nuclear exclusion of Cdc25 caused by Vpr. Overexpression of *srk1* induces cell elongation, an indication of cell cycle G₂ delay, in a similar fashion to Vpr; however, no additive effect of cell elongation was observed when *srk1* and *vpr* were coexpressed, indicating Srk1 and Vpr are likely affecting the cell cycle G₂/M transition through the same cellular pathway. Immunoprecipitation further shows that Vpr and Srk1 are part of the same protein complex. Consistent with our findings in fission yeast, depletion of the *MK2* gene, a human homologue of Srk1, either by small interfering RNA or an MK2 inhibitor suppresses Vpr-induced cell cycle G₂ arrest in mammalian cells. Collectively, our data suggest that Vpr induces cell cycle G₂ arrest at least in part through a Srk1/MK2-mediated mechanism.

Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) is a virion-associated accessory protein with an average length of 96 amino acids and a calculated molecular mass of 12.7 kDa (14). Increasing evidence suggests that Vpr plays an important role in the viral life cycle and pathogenesis of HIV-1. For example, Vpr is required both in vitro and in vivo for efficient viral infection of nondividing cells such as monocytes and macrophages (21–23). Extracellular addition of Vpr to latently infected T lymphocytes markedly increased HIV-1 replication (32). Rhesus monkeys, chimpanzees, and human subjects infected with Vpr-defective viruses demonstrate slow disease progression, often accompanied by reversion of the mutated *vpr* genes back to the wild-type phenotype (17, 19, 30, 56, 65).

Vpr displays several distinct activities in host cells. One of these activities is to lock host cells into the G₂ phase of the cell cycle, known as the G₂ arrest (22, 24, 51, 52). The cell cycle G₂ arrest induced by Vpr is thought to suppress human immune functions by preventing T-cell clonal expansion (50) and to

provide an optimized cellular environment for maximal levels of viral replication (19). Therefore, further understanding of Vpr-induced cell cycle G₂ arrest could provide additional insights into molecular actions of Vpr in augmenting viral replication and modulation of host immune responses.

Vpr induces cell cycle G₂ arrest in eukaryotic cells ranged from fission yeast (*Schizosaccharomyces pombe*) to mammalian cells, suggesting the cellular pathway(s) targeted by Vpr must be highly conserved. Indeed, previous studies have demonstrated that Vpr induces G₂ arrest by promoting hyperphosphorylation of fission yeast Cdc2 or human Cdk1, a kinase that determines onset of mitosis in all eukaryotes (22, 51, 64). Vpr exerts its inhibitory effect through T14 and Y15 of Cdk1 and Y15 of Cdc2, since the expression of nonphosphorylated mutants, T14A Y15F of Cdk1 or Y15F of Cdc2, prevents Vpr-induced G₂ arrest (12, 22). Furthermore, Vpr inhibits the Cdc25 phosphatase (3, 13) and activates Wee1 kinase (13, 58) to promote phosphorylation of Cdc2/Cdk1 during induction of G₂ arrest. Cdc25 normally dephosphorylates Cdc2/Cdk1 to promote mitosis, whereas Wee1 kinase phosphorylates Cdc2/Cdk1 that prevents entry of mitosis. Consistent with the roles of Wee1 and Cdc25 in Vpr-induced G₂ arrest, proteins that are involved in the regulation of Cdc25 or Wee1 have been identified to either enhance or inhibit Vpr-induced G₂ arrest. Fission yeast Wos2, which is a human p23 homologue and a Wee1 inhibitor (45), has been shown to be a multicopy Vpr suppres-

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sor (13). A Cdc25 inhibitor *rad24* (36), which is the human 14-3-3 homologue, enhances Vpr-induced G₂ arrest when overproduced in fission yeast (13). It is thus clear that a regulated balance between the Wee1 kinase and the Cdc25 phosphatase is critical to determine the Cdc2 activity that in turn regulates the G₂/M transition (13, 18, 41).

Upon DNA damage or inhibition of DNA replication, cellular DNA checkpoint responds to these cellular crises by inducing cell cycle G₂ arrest. The G₂ arrest is achieved by shuffling Cdc25 to the cytoplasm, where Cdc25 is no longer able to access Cdc2 and is further degraded by proteolysis (2, 49, 54). In fission yeast, as well as in human cells, the nuclear exclusion of Cdc25 is dependent on Rad24/14-3-3 proteins and Cdc25 phosphorylation, which is mediated through the ATR/ATM-activated Chk1/Chk2 kinases (27, 36, 49, 54, 61). Typically, 14-3-3 binds to phosphorylated Cdc25 that propels it from the nucleus to the cytoplasm, where it is undergoes proteasome-mediated degradation (2, 49, 54). Consistent with this model, a mutant allele of *cdc25* that cannot bind to 14-3-3 proteins or a mutant Cdc25 that cannot be phosphorylated at its nine serine/threonine sites remains nuclear, thus allowing cells to enter mitosis in spite of treatment with agents that activate the mitotic DNA checkpoints (61).

Similar to the regulation of Cdc25 in cellular DNA checkpoint responses, Vpr appears to prevent Cdc25C from entering the nucleus (25). Furthermore, Vpr binds to Cdc25C and 14-3-3 in human cells (18, 26), providing a possible mechanistic basis for the effect of Vpr on Cdc25 and the cell cycle G₂/M regulation. However, whether the upstream kinases such as Chk1 or Cds1/Chk2, which normally phosphorylate Cdc25 during the DNA checkpoints, are specifically responsible for the phosphorylation of Cdc25 and subsequent nuclear exclusion and protein degradation is at present unknown.

Whether Chk1 or Cds1/Chk2 is responsible for the G₂ arrest induced by Vpr is controversial. In fission yeast, mutations in both *chk1* and *cds1*, which are thought to be part of the checkpoints (6, 16, 60), do not block Vpr-induced G₂ arrest (13, 41). However, reports from mammalian studies showed that Vpr activates Chk1 for the G₂ induction (67, 68). Considering that mitotic DNA checkpoints are highly conserved between mammalian and fission yeast cells, it is unclear at the moment why, given that activation of human Chk1 by Vpr is at least partially required for G₂ arrest, deletion of *chk1*, *cds1*, or *chk1/cds1* (homologues of Chk1/Chk2) does not block Vpr-induced G₂ arrest in fission yeast (12, 13). One possibility is that other kinase(s), i.e., other than Chk1 or Cds1/Chk2, are involved in the phosphorylation of Cdc25 during the G₂ induction by Vpr.

The fission yeast Srk1 kinase (for Sty1-regulated kinase 1) and its mammalian counterpart, MAPKAP kinase-2 (MK2), have recently been implicated as a third possible kinase, in addition to Chk1 and Chk2, to regulate Cdc25 (35, 38). The Srk1 phosphorylates Cdc25 by direct interaction at the same phosphorylation site as Chk1 and Cds1, and that overexpression of *srk1* causes cell cycle arrest in a cell lacking both Cds1 and Chk1 (35). Importantly, Srk1 does not regulate Cdc25 in response to the DNA damage or replication checkpoints but only under the normal growth conditions or in response to nongenotoxic environmental stress (35). Similarly, Ser216 of Cdc25C has also been shown to be the optimal phosphorylation site by MK2 and depletion of MK2 by small interfering

TABLE 1. Fission yeast strains

Strain	Host genotype	Source or reference
SP223	<i>h⁻ ade6-216 leu1-32 ura4-294</i>	Lab stock
RA112	<i>h⁻ srk1::kanMX6 leu1-32 ura4-D18</i>	35
RE007	<i>h⁻ ade6-216 leu1-32 ura4-294::vpr(NL4-3)::ura4⁺</i>	4
RE076	<i>h⁻ ade6-216 leu1-32 ura4-294::vpr(F34I)::ura4⁺</i>	13
RE109	<i>h⁻ cdc25GFPint cdc25::ura4⁺ leu1-32 leu2⁺ ura4-294::vpr(NL4-3)::ura4⁺</i>	This study
Q2016	<i>h⁻ cdc25GFPint cdc25::ura4⁺ leu1-32 leu2⁺</i>	7
Q2019	<i>h⁺ cdc25GFPint cdc25::ura4⁺ rad24::ura4⁺ leu1-32 leu2⁺</i>	7
RE285	<i>cdc25GFPint cdc25::ura4⁺ rad3-136 leu1-32 leu2⁺ ura4-294::vpr(NL4-3)::ura4⁺</i>	This study
SH2	<i>leu1-32 ura4-294::vpr(F34I)::ura4⁺ srk1::kanMX6</i>	This study
SH3	<i>cdc25GFPint cdc25::ura4⁺ rad24::ura4⁺ leu1-32 leu2⁺ kanr::vpr(NL4-3)::ura4-294</i>	This study
SH4	<i>chk1::ura4⁺ leu1-32 kanr::vpr(NL4-3)::ura4-294</i>	This study
SH5	<i>cds1::ura4⁺ chk1::ura4⁺ leu1-32 kanr::vpr(NL4-3)::ura4-294</i>	This study
SH6	<i>cds1::ura4⁺ leu1-32 ura4-294::vpr(NL4-3)::ura4⁺</i>	This study

RNA (siRNA) abolished UV-induced Cdc25C phosphorylation (38).

Because of the controversy regarding the involvement of Chk1/Chk2 and the other regulators of Cdc25, it is possible that Vpr may inhibit Cdc25 by phosphorylation through a different kinase such as Srk1/MK2. Thus, a more detailed knowledge of the mechanisms involving Cdc25 should provide new insight about the cellular response to *vpr* gene expression during the induction of cell cycle G₂ arrest. The goal of the present study was to use the fission yeast model to further delineate the molecular regulation of Cdc25 in response to *vpr* gene expression. We hypothesized that Vpr induces cell cycle G₂ arrest at least in part through an Srk1/MK2-mediated regulatory pathway. The results described here confirm this hypothesis. Our data in fission yeast have further shown that, as in mammalian cells and the DNA checkpoints, Vpr also promote nuclear exclusion of Cdc25 through a Rad24/14-3-3-dependent mechanism. However, Chk1/Cds1, the fission yeast homologues of human Chk1 and Chk2, are not involved in the Cdc25 nuclear exclusion. Instead, Srk1/MK2 is one of the kinases modulated by Vpr to inhibit Cdc25 for the induction of cell cycle G₂ arrest.

MATERIALS AND METHODS

Yeast strains, media and genetic manipulations. The *S. pombe* strains used in the present study are listed in Table 1. Strains were grown in complete medium containing adenine (YEA) or Edinburgh minimal medium (EMM) by using standard culture techniques (44). Standard genetic and biochemical methods for studying fission yeast were followed as described previously (44). Conjugation and sporulation were performed on SPAS mating plates, followed by the random spore analysis (44). Briefly, 10⁵ cells of two opposite mating type strains were mixed together, and the total cells were spotted onto the SPAS plates. Formation of four-spore containing asci was seen under the light microscope after 2 to 3 days of incubation at 30°C. A total of 1 ml of sterile distilled water was inoculated with a loopful of the cross, and glucosylase (Perkin-Elmer) was added at a final concentration of 0.5%. The mixture was incubated for 6 h at 30°C. Then, 1,000 spores were plated out on the uracil (75 µg/ml) containing YEA (YEAU)

medium. The plates were then incubated at 30°C, and the colonies were analyzed on the EMM plates containing appropriate supplements. Genetic deletion of a specific allele in different strains was also validated by PCR with sequence-specific primer pairs.

Cell growth and gene expression in fission yeast cells. Gene induction under the control of the fission yeast *nmf1* (for no message in thiamine 1) promoter in liquid medium has been previously described (43, 64). Cells containing *nmf1* promoter plasmid were maintained in appropriately supplemented EMM with 20 μ M thiamine. For gene induction, cells were first grown to mid-log-growth phase in the presence of 20 μ M thiamine. Cells were then washed three times with distilled water and diluted at a final concentration of approximately 2×10^5 cells/ml in 5 ml of appropriately supplemented EMM with (gene-off) or without (gene-on) thiamine. All cells were normally grown at 30°C with constant shaking at 200 rpm.

Fluorescence microscopy. A Leica DMR fluorescence microscope (DM4500B; Leica Microsystems) equipped with a high-performance camera (Hamamatsu) and an OpenLab software (Improvision) was used for all of the imaging analysis. For measuring the subcellular localization of green fluorescent protein (GFP)-tagged Cdc25 fusion proteins in fission yeast, live cells were observed under a fluorescence microscope, and images were captured 18 to 20 h after *vpr* gene induction. The location of Cdc25 was detected by emission of the green fluorescent signals, and the nuclear compartmentation was confirmed by DNA staining with 1 μ g of DAPI (4',6'-diamidino-2-phenylindole)/ml. To block nuclear export of proteins, leptomycin B (LMB) was added to the culture at a final concentration of 20 ng/ml, and the cells were then visualized 1 h later as described previously (61). Cells were also treated with cisplatin, a DNA-damaging agent (29), at a final concentration of 0.5 mM, and they were visualized 4.5 h later. To measure cell length, cell images were first captured, and the cell length was measured individually as previously described (4, 13) with the captured images by using the OpenLab software according to the manufacturer's instructions.

Cell lysis and immunoblotting analysis. For fission yeast, cell lysates were prepared by the glass beads method using lysis buffer (50 mM Tris-HCl [pH 8], 50 mM NaF, 1 mM Na_3VO_4 , 5 mM EDTA, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) supplemented with protease inhibitors as previously described (4). For mammalian cells, the cell lysates were prepared by resuspending the cells in lysis buffer (50 mM Tris-HCl [pH 7.5], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) also supplemented with protease inhibitors as described previously (4, 33). All cell extracts were centrifuged for 20 min at 14,000 rpm. The supernatants were removed and placed into new tubes. The protein concentration was calculated by using a BCA protein assay kit (Pierce). In some cases, proteins were immunoprecipitated from the cell lysates with the indicated antibody overnight at 4°C. The protein-antibody complexes were subsequently collected by adding protein A-agarose beads, and immunoprecipitates were washed three times with phosphate-buffered saline (PBS) buffer containing protease inhibitors prior to analysis.

For Western blot analysis, 25 to 50 μ g of proteins was electrophoresed through 10 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels (premade by Bio-Rad) and electrophoretically transferred to nitrocellulose membranes. Membranes were incubated for 60 min at room temperature in blocking buffer (TBST-5% milk). Membranes were then incubated overnight at 4°C with the primary antibody diluted in blocking buffer (TBST-2% milk). The immunoblots were washed three times in TBST and incubated with the secondary antibody for 60 min at room temperature. The immunoblots were washed again three times in TBST, and the proteins were visualized by using the chemiluminescence detection kit (Pierce). The following primary antibodies were used: mouse monoclonal anti-hemagglutinin (anti-HA; HA-7) antibody (dilution, 1/10,000; Sigma), mouse monoclonal anti- β -actin (AC-15) antibody (dilution, 1/5,000; Sigma), rabbit polyclonal anti-MAPKAPK-2 antibody (dilution, 1/1,000; Cell Signaling), rabbit polyclonal anti-Cdc25C Ser216 (dilution, 1:500; Cell Signaling), and mouse monoclonal anti-Cdc25C (0.5 μ g/ μ l; Upstate); rabbit polyclonal anti-Vpr serum (dilutions, 1/300 or 1/500 as indicated) was custom generated by the Proteintech Group, Inc. (Chicago, IL). Goat anti-mouse horseradish peroxidase-conjugated and goat anti-rabbit horseradish peroxidase-conjugated antibodies were used as secondary antibodies (dilution, 1/5,000; Bio-Rad).

Mammalian cell culture, siRNA transfection, and *vpr* expression. The DL-3 cell line, generated in our laboratory (4), is a derivative of the HEK293VE-632 cell line that is stably transfected with an inducible *vpr* expression plasmid (pZH-*vpr*) (66). DL-3 cells were maintained in Dulbecco modified Eagle medium (Cellgro) supplemented with 10% fetal bovine serum (Invitrogen) and 100 μ g of zeocin and 200 μ g of hygromycin B (both from Invitrogen)/ml. The expression of *vpr* was induced by 1 μ M muristerone A (Invitrogen) for 72 h as described previously (4, 66). The anti-MK2 siRNA, which was designed and

validated to specifically silence the expression of the MK2 gene (GenBank accession numbers NM_004759 and NM_032960), was purchased from Ambion (catalog no. AM51331; ID no.1598). The siRNA-MK2 was transfected at a concentration of 20 nM into approximately 10^6 dividing DL-3 cells by using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen). At 24 h posttransfection (p.t.), DL-3 cells were treated with muristerone A to induce *vpr* expression. Cells were harvested 24, 48, or 72 h p.t. for cell growth, observation of cell morphology, and immunoblotting analyses. For flow cytometric analysis, the same cells were collected at 72 h p.t. As an alternative procedure, approximately 10^6 dividing DL-3 cells were also treated with a selective MK2 inhibitor (MK2a; Calbiochem; catalog no. 475863), which interrupts p38 α -mediated MK2 phosphorylation (10, 37) at a final concentration of 50 μ M. At 24 h after the MK2a treatment, the existing medium was replaced by fresh medium containing MK2a at 50 μ M, and the expression of *vpr* was then induced with muristerone A for 72 h.

Cell cycle analysis by flow cytometry. Cells were treated with trypsin and washed twice with 5 mM EDTA-PBS buffer. Cells were resuspended and fixed with 70% cold ethanol, and they were incubated for 10 min on ice. Fixed cells were washed twice with 5 mM EDTA-PBS buffer and diluted at a final concentration of 10^6 cells/ml in PBS buffer. The fixed cells were then incubated with RNase A (5.0 μ g/ml) for 30 min at 37°C and placed on ice for 60 min after the addition of propidium iodide (10 μ g/ml). DNA profiles of the propidium iodide-stained cells were analyzed on a Becton-Dickinson FACScan, the data were analyzed, and cell cycle plots were generated by using ModFit software (Verity Software House).

Other molecular and cellular techniques. The growth dynamics of HEK293 cells with or without the MK2 knockdown was determined by measuring cell growth. A total of 10^5 cells were initially inoculated, and the cell numbers were manually counted under the microscope by using a hemacytometer. Possible cell death caused by the MK2 knockdown was evaluated by trypan blue staining, a dye that specifically recognizes dead cells (Sigma). The doubling time of each cell population was then calculated. The images of these cells were captured at the indicated times by using a Leica microscope to document the cell morphology.

RESULTS

Vpr promotes cytoplasmic compartmentalization of Cdc25 through serine/threonine phosphorylation of Cdc25. Earlier studies showed that Vpr induces cell cycle G_2 arrest in part by inhibiting the Cdc25 phosphatase activity both in fission yeast and in mammalian cells (13, 18). Mammalian data further showed that Vpr binds directly to Cdc25C and 14-3-3, which typically binds to Cdc25C when cellular checkpoint controls are activated (49, 61). However, whether the binding of Vpr to Cdc25C or the inhibitory effect of Vpr on the Cdc25C phosphatase activity is required for Vpr-induced G_2 arrest is not clear at present since both the catalytic active Cdc25C with reduced binding to Vpr and the catalytic inactive Cdc25C that binds to Vpr are able to overcome Vpr-induced G_2 arrest (18). Interestingly, another study in mammalian cells further showed that Vpr facilitates redistribution of Cdc25C into the cytoplasm (25). It is thus conceivable that Vpr may inhibit Cdc25 by limiting the access of Cdc25 to Cdc2 in the nucleus, thereby resulting in the hyperphosphorylation of Cdc2. Here we were interested to determine whether Vpr has a similar effect on Cdc25 subcellular redistribution in *S. pombe*.

To facilitate the examination of Cdc25 cellular distribution, the Cdc25 protein was fused with a GFP at its N-terminal end as previously described (36). It has been demonstrated previously that the *gfp-cdc25* expression is capable of rescuing the *cdc25-22* mutant; thus, the GFP tag does not affect the Cdc25 functions (61). To ensure the GFP-Cdc25 behaves the same way as previously described, the GFP-Cdc25 subcellular localization was first monitored in wild-type strain without *vpr* gene expression (Fig. 1A). The plasmids DNA containing the *gfp-cdc25* fusion or *gfp* by itself were transformed into the wild-

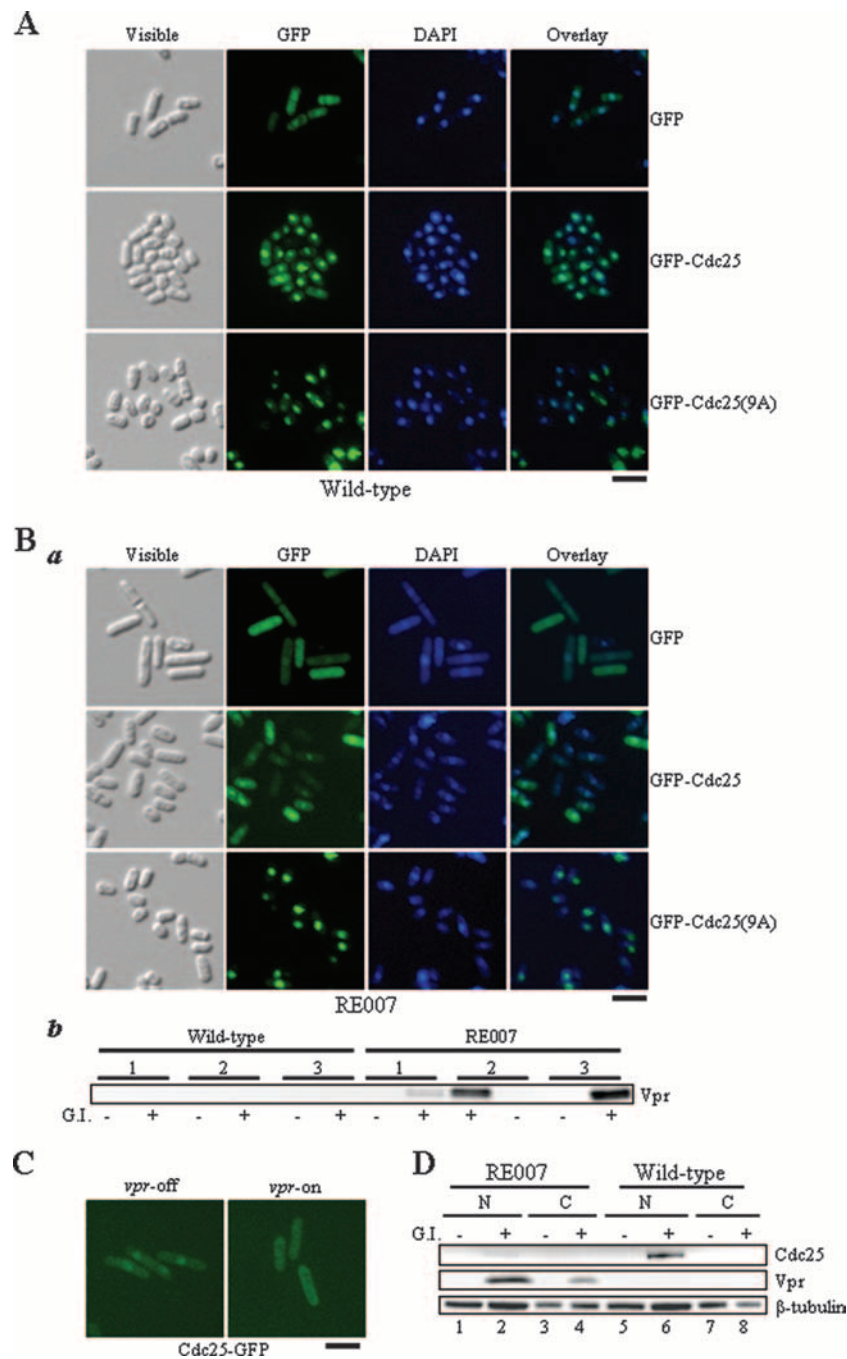


FIG. 1. Vpr promotes cytoplasmic compartmentalization of Cdc25 through serine/threonine phosphorylation of Cdc25. The wild-type SP223 strain (A) and the RE007 (B) yeast strain, which is a derivative of the SP223 containing a single integrated copy of the *vpr* gene under the control of the *nmr1* promoter, were transformed with pREP41x-GFP, pREP41x-GFP-*cdc25*, or pREP41x-GFP-*cdc25*(9A). To visualize the subcellular localization of Cdc25, these strains were grown in liquid selective medium in the absence of thiamine for 20 h to induce gene expression. (Ba) All of the cells were collected 20 h after gene induction and visualized microscopically. Cdc25 was detected by the emission of green fluorescence, and nuclear DNA was stained with DAPI. Bar, 10 μ m. (Bb) Immunoblot analysis was performed in the test strains to confirm the absence or expression of *vpr* in the wild type or the RE007 strains after transformation with the plasmids of pREP41x-GFP, pREP41x-GFP-*cdc25*, or pREP41x-GFP-*cdc25*(9A), labeled 1, 2, and 3, respectively. (C) The RE109 yeast strain, which is derived from RE007 strain carrying an integrated copy of the *cdc25-gfp* gene, was grown in liquid culture for 20 h in the presence (*vpr*-off) or in the absence (*vpr*-on) of thiamine to induce *vpr* gene expression. The *cdc25-gfp* gene is constitutively expressed under its native promoter (7). Immunoblot analysis was carried out and confirmed the expression of *vpr* in the RE109 strain (data not shown). For all of the immunoblot analyses, *vpr*-repressing and *vpr*-expressing cells were collected and lysed 20 h after gene induction. Equal amounts of proteins (25 μ g) were loaded onto a 10 to 20% gradient SDS-PAGE gel. Vpr was visualized with anti-Vpr (dilution, 1/500). (D) Vpr promotes protein degradation of Cdc25. Wild-type (SP223) and RE007 yeast strains transformed with pREP41x-GFP-Cdc25 were grown in liquid culture for 24 h in the presence or absence of thiamine to induce gene expression. Cell fractionation was performed to obtain cytoplasmic (C) and nuclear (N) extracts from the cells. A Western blot analysis was carried out to detect Vpr and Cdc25. G.I., gene induction.

type SP223 *S. pombe* cells. Both of these two proteins were produced under the control of a thiamine repressible *nmt1* promoter (43) and cellular location of Cdc25 in live fission yeast cells collected 20 h after gene induction were visualized by microscopy. Consistent with the earlier studies (36, 61), the GFP-Cdc25 protein was found in both the cytoplasm and the nucleus of the cells, with relative higher concentration of GFP-Cdc25 in the nucleus (Fig. 1A, middle row). Nuclear accumulation of the Cdc25 was detected in $75\% \pm 3\%$ of the cells expressing *gfp-cdc25*. The cells were further stained with DAPI for nuclear DNA, and the overlapping images between green fluorescent signals and DAPI staining confirmed the abundant presence of GFP-Cdc25 in the nucleus (Fig. 1A, middle row). In contrast, GFP alone dispersed throughout the cell (Fig. 1A, top row).

To test the effect of *vpr* gene expression on subcellular localization of GFP-Cdc25, the same experiments were carried out in the RE007 strain, which is a derivative of the wild-type SP223 but contains a single integrated copy of the *vpr* gene in its chromosome (4). At 20 h after *vpr* gene induction, the subcellular location of the GFP-Cdc25 was examined by fluorescence microscopy (Fig. 1Ba). Different from the nuclear presence of Cdc25 in the wild-type cells, the GFP-Cdc25 was predominantly localized in the cytoplasm in the *vpr*-expressing cells (Fig. 1Ba, middle row). Nuclear GFP-Cdc25 was detected only in $20\% \pm 3\%$ of the *vpr*-expressing cells compared to $75\% \pm 3\%$ of the cells without Vpr (Fig. 1A, middle row). Regardless of the presence of Vpr, the localization of GFP in RE007 strain remained dispersed throughout the cell, suggesting that the redistribution of Cdc25 observed in the *vpr*-expressing cells is Cdc25 specific (Fig. 1Ba, top row).

To avoid the potential artifacts induced by the ectopic expression of *gfp-cdc25* or the GFP tagging, the Vpr-mediated redistribution of Cdc25 was further verified by using a yeast strain RE109 that carries a single integrated copy of *cdc25-gfp* gene in the *cdc25*⁺ genomic locus. In addition, the *cdc25* gene was tagged with *gfp* at its C terminus and expressed from the genome under its own endogenous promoter of *cdc25* as previously reported (7). The RE109 strain was generated by genetic crossing between Q2019 and RE007; thus, it also carries a single integrated copy of the *vpr* gene in the chromosome as the RE007. Similar to what we observed in cells, when the GFP-Cdc25 was ectopically produced, the Cdc25-GFP was also found predominantly ($80\% \pm 6\%$) nuclear in the *vpr*-repressing cells (Fig. 1C, left). Upon *vpr* expression, most of the cells had cytoplasmic Cdc25-GFP; only 9% of them had nuclear Cdc25-GFP (Fig. 1C).

To confirm Vpr-mediated nuclear export of Cdc25, we carried out Western blot analysis to compare the protein levels of Cdc25 in the cytoplasm and nuclear compartments using the cells shown in Fig. 1B. Careful comparison of the Cdc25 protein levels in cells with or without *vpr* gene expression revealed that Cdc25 might have been degraded in the *vpr*-expressing cells. As shown in Fig. 1D, Cdc25 is mainly nuclear in the wild-type cells without Vpr (Fig. 1D, lane 6). No Cdc25 was detected in the cytoplasm compartment (Fig. 1D, lane 8). When the Cdc25 protein levels were remeasured in the nuclear and cytoplasm fractions, however, a reduced Cdc25 protein level was seen in the nucleus, and no Cdc25 was detected in the cytoplasm, indicating potential protein degradation of Cdc25

once it is redistributed into the cytoplasm (Fig. 1D, lane 4). This is in sharp contrast with cells without Vpr, in which Cdc25 was mainly nuclear (Fig. 1D, lane 6). Therefore, this observation suggests that Vpr may promote protein degradation of Cdc25 once it enters the cytoplasm.

Cell cycle G₂ arrest and nuclear export of Cdc25 are also observed typically in cells with activated mitotic DNA checkpoints. In response to DNA damage or inhibition of DNA replication, Cdc25 is first being phosphorylated at the serine/threonine sites, followed by binding to Rad24, which escorts Cdc25 to the cytoplasm (15, 36, 61). A mutant of Cdc25 protein, Cdc25(9A), which contains alanine in place of the nine possible serine/threonine phosphorylation sites, has been shown to severely impair its ability to bind Rad24. Consequently, it remains in the nucleus regardless of the cellular checkpoint responses (61).

To investigate whether the phosphorylation of Cdc25 at these sites is also involved in the nuclear export of Cdc25 induced by Vpr, the RE007 strains was transformed with plasmid DNA carrying a *gfp-cdc25(9A)* gene, and the subcellular localization of GFP-Cdc25(9A) was compared to that of the wild-type *gfp-cdc25* gene in the presence or absence of Vpr. Significantly, the subcellular localization patterns of the mutant Cdc25 were essentially the same regardless of the absence or presence of Vpr (Fig. 1A, bottom row, versus Fig. 1Ba, bottom row). The GFP-Cdc25(9A) accumulated predominantly in the nucleus of $95\% \pm 2\%$ of both types of cells, suggesting that the wild-type GFP-Cdc25 is actively exported from the nucleus to the cytoplasm by Vpr, possibly via phosphorylation at the serine/threonine sites of Cdc25.

Vpr-mediated nuclear exclusion of Cdc25 is Rad24 and Crm1 dependent. In cells with activated DNA checkpoint controls, Chk1 or Cds1 phosphorylates Cdc25, which creates a binding site for Rad24 that escorts Cdc25 from the nucleus to the cytoplasm (61). Because phosphorylation of Cdc25 is required for nuclear export induced by Vpr, we were interested in testing whether Vpr-mediated nuclear-cytoplasmic shuttling also relies on Rad24. To address this question, subcellular distribution of Cdc25 was examined in a *rad24* deletion mutant (SH3) in the presence or absence of Vpr. The fission yeast strain SH3, which carries a deletion of *rad24* gene and a single integrated copy of *vpr* and Cdc25-GFP genes, was generated by genetic crossing between Q2019 and RE109 (Fig. 2A). As a control, the subcellular distribution of Cdc25-GFP was also monitored in a wild-type *vpr*-expressing RE109 strain (Fig. 2A, top row, and Fig. 2B). Consistent with our observation shown in Fig. 1C, upon *vpr* expression the Cdc25-GFP redistributed from the nucleus to the cytoplasm (Fig. 2A, top row). In contrast, in the $\Delta rad24$ mutant strain Cdc25 was retained in the nucleus regardless of whether the *vpr* gene is expressed (Fig. 2A, bottom row), suggesting that the cytoplasmic redistribution of Cdc25 induced by Vpr is indeed dependent on Rad24. The inability of Cdc25 to exit the nucleus in the $\Delta rad24$ strain was further confirmed by examining its localization in the Q2019 strain, in which $96\% \pm 1\%$ of the *rad24* deletion cells (Fig. 2A, middle row) was observed with nuclear Cdc25-GFP (61).

To further examine whether nuclear export of Cdc25 induced by Vpr is mediated through an active nuclear import-export process, the subcellular distribution of Cdc25-GFP in

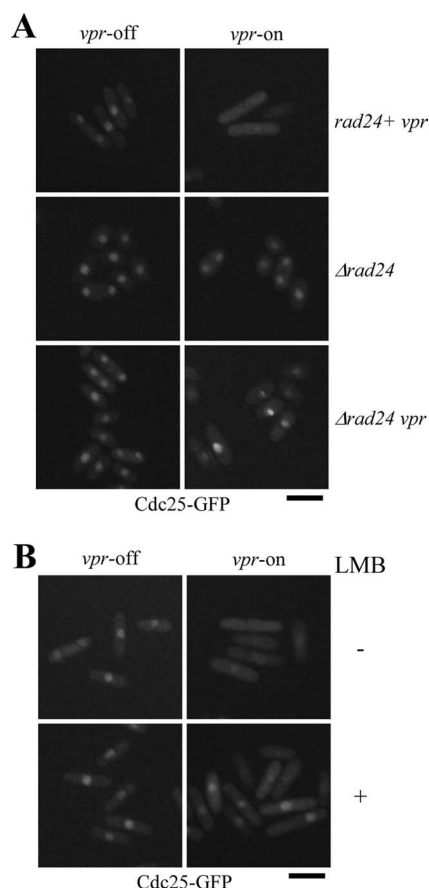


FIG. 2. Vpr-mediated nuclear exclusion of Cdc25 is Rad24 and Crm1 dependent. (A) Rad24-dependent nuclear exclusion. The RE109 (*rad24⁺ vpr*), Q2019 ($\Delta rad24$), and SH3 ($\Delta rad24 vpr$) fission yeast strains were grown in liquid culture for 20 h either in the presence (*vpr-off*) or in the absence (*vpr-on*) of thiamine to induce gene expression. Immunoblot analysis was performed to confirm the proper expression of *vpr* in RE109 and SH3 cells. The same levels of Vpr proteins were expressed in each of these samples (data not shown). (B) Treatment of *vpr*-expressing cells with the nuclear export Crm1 inhibitor LMB blocks the Vpr-mediated nuclear exclusion of Cdc25. The RE109 fission yeast strain was grown in liquid culture for 20 h either in the presence (*vpr-off*) or in the absence (*vpr-on*) of thiamine to induce gene expression. When indicated, LMB was added to the culture at a final concentration of 20 ng/ml as previously described (4, 61), and cellular localization of Cdc25 were performed 1 h after the treatment. Immunoblot analysis was performed to confirm the proper expression of *vpr* in the RE109 cells either in the presence or in the absence of LMB. For the immunoblot analyses, all cells were collected and lysed 20 h after gene induction. Equal amounts of proteins (25 μ g) were loaded onto SDS-PAGE gels, and the Vpr was visualized with anti-Vpr (dilution, 1/500). The same levels of Vpr proteins were expressed in each of these samples (data not shown). Subcellular localization of Cdc25-GFP was visualized microscopically by the emission of green fluorescence. Bar, 10 μ m.

the presence or absence of Vpr was monitored in RE109 cells treated with LMB, a specific inhibitor of Crm1 (47) that is a key protein involved in the nuclear export of proteins (Fig. 2B). The expression of *vpr* was first induced for 20 h in the RE109 cells, and these cells were then treated with LMB for 1 h as described previously (4, 61). As shown in Fig. 2B (left side), the wild-type cells with or without LMB treatment showed a sim-

ilar and predominant presence of Cdc25 in the nucleus. Approximately $81\% \pm 1\%$ of the cells show nuclear Cdc25-GFP without treatment (Fig. 2B, top left) versus ca. $95\% \pm 1\%$ in the LMB-treated cells (Fig. 2B, bottom row). Even though Cdc25 in most of the *vpr*-expressing cells is uniformly distributed throughout the cells without LMB (Fig. 2B, top right), treatment of the *vpr*-expressing cells with LMB prevented the nuclear export of Cdc25-GFP, resulting in the pronounced presence of nuclear Cdc25. About $50\% \pm 4\%$ of the LMB-treated *vpr*-expressing cells was then detected with Cdc25-GFP in the nucleus (Fig. 2B, bottom row). Therefore, the nuclear export of Cdc25 by Vpr is likely mediated through an active nuclear-cytoplasm shuttling via Crm1.

Cells defective in DNA damage and DNA replication checkpoints do not affect the Vpr-mediated nuclear export of Cdc25. Since Vpr keeps Cdc25 out of the nucleus in a way similar to the classic mitotic checkpoint responses, the possibility that Vpr might regulate the activity of Cdc25 through cellular components of the same checkpoint signaling pathway was evaluated. Rad3 kinase, the human homologue of ATM/ATR, is a critical component of both the DNA replication and the DNA damage checkpoints in *S. pombe*. The subcellular distribution of Cdc25-GFP under the *vpr*-expressing conditions was examined in a fission yeast strain RE285 that carries the *rad3-136* mutation and single integrated *vpr* and *cdc25-gfp* genes. The lack of the checkpoint response in the RE285 strain was first tested by treating them with cisplatin (Fig. 3A), a DNA-damaging agent (29). As a control, the wild-type Q2016 cells expressing *cdc25-gfp* was also treated with cisplatin. Consistent with the idea that cisplatin triggers DNA damage checkpoint resulting in cell cycle G₂ arrest, cisplatin-treated wild-type cells became much elongated (Fig. 3A, top row), an indication of cell cycle arrest (31, 48). In contrast, cisplatin-treated *rad3-136* cells did not show elongated phenotype, indicating loss of the checkpoint response (Fig. 3A, bottom row). Consistent with these phenotypes and previous reports (36, 61), treatment of the wild-type cells with cisplatin caused nuclear exclusion of Cdc25 (Fig. 3A, top row). Conversely, the *rad3-136* mutation blocked cisplatin-mediated nuclear export of Cdc25 (Fig. 3A, bottom row). Approximately $81\% \pm 3\%$ of the cisplatin-treated *rad3-136* cells was observed with a strong nuclear Cdc25-GFP compared to $3\% \pm 1\%$ of the cisplatin-treated wild-type cells (Fig. 3A). When the *vpr* gene was expressed in these strains, however, the localization pattern of Cdc25-GFP was found to disperse throughout the cells regardless of the cells are wild-type or *rad3-136* (Fig. 3A). Therefore, genetic defect in the checkpoint *rad3* gene does not appear to affect the ability of Vpr to expel Cdc25 to the cytoplasm.

The kinase Srk1, instead of Chk1 or Cds1, is required by Vpr for the nuclear export of Cdc25. Since Rad3 is an early checkpoint protein, we next tested whether Chk1 or Cds1, which normally phosphorylate Cdc25 as the late steps of the DNA checkpoint controls, are involved in the Vpr-mediated nuclear export of Cdc25. The $\Delta chk1$ (SH4), $\Delta chk1/\Delta cds1$ (SH5), and $\Delta cds1$ (SH6) fission yeast strains, all of which also carry a single integrated copy of the *vpr* gene (13), were transformed with the expression vector pREP41x-*gfp-cdc25*, and the subcellular distribution of GFP-Cdc25 was examined under the influence of Vpr. Similar to what we observed in the *rad3-136* mutant strain, the $\Delta chk1$ and/or $\Delta cds1$ deletions had no effect

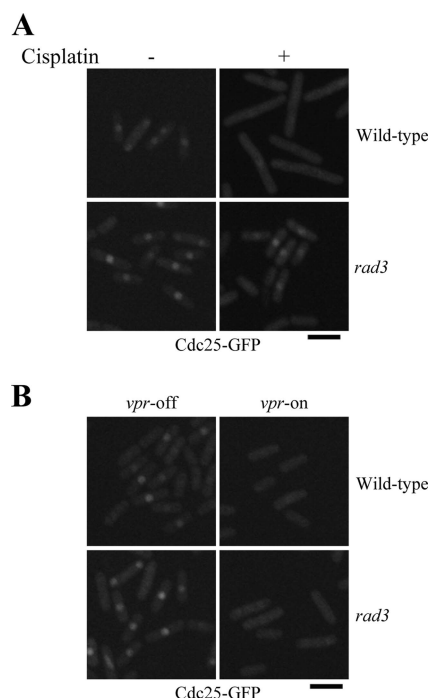


FIG. 3. Fission yeast cells defective in DNA damage or DNA replication checkpoints do not affect Vpr-mediated nuclear export of Cdc25. The *rad3-136* mutation blocks nuclear export of Cdc25 induced by cisplatin (A) but not that induced by Vpr (B). The wild-type (Q2016) and *rad3-136* (RE285) fission yeast strains were grown in liquid culture for 20 h in the presence of thiamine. When indicated, cisplatin was added to the culture at a final concentration of 0.5 mM, and the cell size and subcellular location of Cdc25 were determined 4.5 h after treatment. The wild-type (RE109) and *rad3-136* (RE285) fission yeast strains were grown and assayed the same way as described above. Bar, 10 μ m.

on Vpr-mediated nuclear export of GFP-Cdc25 (Fig. 4A), suggesting that the effect of Vpr on nuclear-cytoplasmic shuttling of Cdc25 is independent of these major checkpoint control kinases.

That the *rad3-136*, Δ *chk1*, and Δ *cds1* mutations are capable of blocking nuclear export of Cdc25 induced by checkpoints (35, 60) but do not block nuclear export of Cdc25 induced by Vpr suggests that the regulatory effect of Vpr on Cdc25 is not mediated primarily through the DNA damage or DNA replication checkpoints. Since Vpr-mediated nuclear export of Cdc25 required serine/threonine phosphorylations (Fig. 1Ba), kinase(s) other than Chk1 or Cds1 must be involved in regulating the inhibitory phosphorylation of Cdc25 induced by Vpr. One recent report led us to believe that *Srk1* might be a candidate (35). In that report, it was shown that *Srk1* acts as a negative regulator of the G_2 /M transition. Specifically, under normal cell cycle conditions or cellular response to osmotic stress, *Srk1* negatively controls mitotic entry by direct and inhibitory phosphorylation of Cdc25 (35). Moreover, *Srk1* phosphorylates Cdc25 on some of the same residues that Chk1 and Cds1 phosphorylate during cellular checkpoint responses, and these phosphorylations are necessary for *Srk1* to induce the cell cycle G_2 arrest (35). Therefore, the possibility that Vpr may promote the nuclear export of Cdc25 through *Srk1* was evaluated by observing the subcellular localization GFP-Cdc25

in the Δ *srk1* yeast strain transformed with the expression vectors of pREP41x-*gfp-cdc25* and pYZ2N-*vpr* (Fig. 4A). As shown in (Fig. 4A, bottom row), GFP-Cdc25 remained primarily in the nucleus in spite of the *vpr* gene expression. Western blot analysis further confirmed proper production of Vpr in this strain (data not shown). This finding is in contrast to the wild-type control, wherein Vpr induced a strong cytoplasmic compartmentalization of GFP-Cdc25 (Fig. 4A, top row). To test whether the Δ *srk1* mutation itself affects localization of Cdc25, plasmids containing GFP, GFP-Cdc25, or GFP-Cdc25(9A) were introduced into the RA112 strain, and subcellular localizations of these proteins were observed under a fluorescence microscope. Regardless of whether the cells were *srk1*⁺ or Δ *srk1*, both GFP-Cdc25 and GFP-Cdc25(9A) localized principally in the nucleus (Fig. 4B), suggesting that the deletion of *srk1* gene has no noticeable effect on Cdc25 under normal conditions. Together, these findings suggest that *Srk1* is required by Vpr to promote the nuclear export of Cdc25.

Vpr induces cell cycle G_2 arrest through association with *Srk1*. Overexpression of *srk1* was shown to induce cell cycle G_2 arrest, as indicated by greatly increased cell length and measurement of the DNA content (35). To verify this phenotype, we also expressed *srk1*⁺ and kinase-defective *srk1*(K153A) mutants in wild-type SP223 cells. As shown in Fig. 5A (top row) and consistent with the Lopez-Aviles's report, a greatly elongated cell phenotype was observed compared to *srk1*-repressing cells (38.9 ± 4.4 μ m versus 10.3 ± 1.4 μ m). In contrast, significant reduction of the *Srk1*-induced cell elongation (18.0 ± 0.5 μ m) was seen in cells expressing the kinase-defective *srk1*(K153A) mutant. We noticed that, compared to the *srk1*(K153A)-repressing cells, *Srk1*(K153A) still caused significant cell elongation (18.0 ± 0.5 versus 10.1 ± 1.7 μ m). This may indicate a residual *Srk1* kinase activity of this mutant (Fig. 5A, bottom row).

In order to functionally link the role of *Srk1* to Vpr-induced cell cycle G_2 arrest, the possible effect of *srk1* expression on Vpr-induced cell elongation (13, 41) was evaluated. A fission yeast strain RE076 that carries in its genome a single integrated copy of *vpr*(F34I) gene under the control of the *nmt1* promoter was used to test the *Srk1* effect (13). This Vpr mutant, in which phenylalanine was replaced with isoleucine at position 34 (F34I), was used here because Vpr(F34I) does not cause cell death like the wild-type Vpr, but it induces cell elongation, a hallmark of the G_2 arrest and/or delay that can be readily measured (4, 5, 13, 41). As shown in Fig. 5A, expression of *vpr*(F34I) in the RE076 strain induced cell elongation that is typical of a cell cycle G_2 delay (4, 13). Cell length measurement 27 h after gene induction indicated that *vpr*-expressing cells were longer than *vpr*-repressing cells (17.2 ± 0.2 μ m versus 10.3 ± 1.1 μ m), which is consistent with our previous observations (4, 13). Given that both *Srk1* and Vpr induce cell elongation, we tested the potential additive effect of these two proteins when the two genes were coexpressed. If an additive effect were observed, it would suggest that Vpr and *Srk1* exert their effects in separate and independent regulatory pathways. If no additive cell elongation were detected, it would indicate that Vpr and *Srk1* may work in concert in the same regulatory pathway for Vpr-induced cell cycle G_2 arrest. As shown in Fig. 5A, even though the expression of *vpr*(F34I) or *srk1* caused significant cell elongation (17.2 ± 0.2 μ m and 38.9 ± 4.4 μ m,

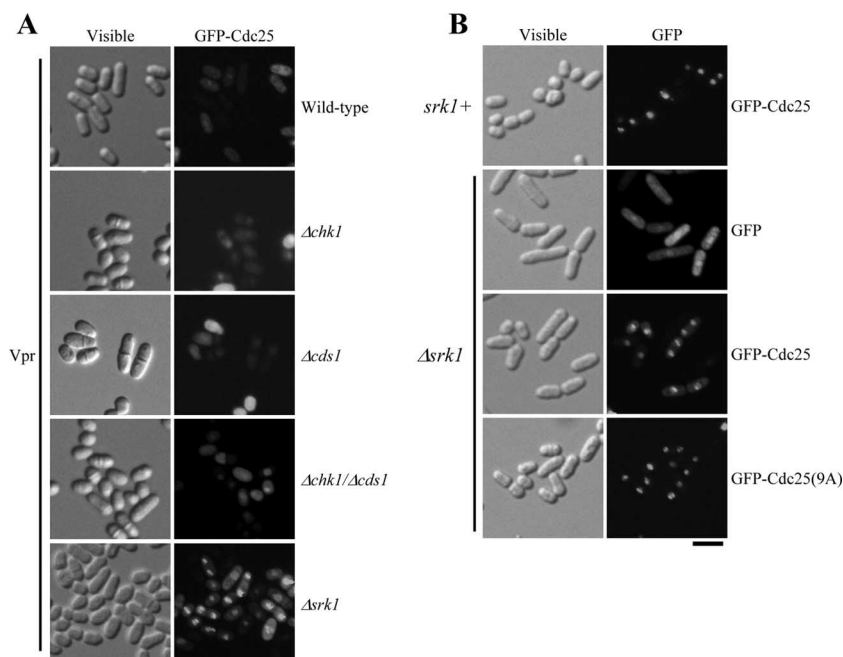


FIG. 4. Kinase Srk1, instead of Chk1 or Cds1, is required by Vpr for the nuclear export of Cdc25. (A) Deletions of the *chk1*, *cds1*, or *chk1/cds1* genes are unable to block Vpr-mediated nuclear export of Cdc25, but deletion of the *srk1* gene abrogated the Vpr effect on Cdc25. All of the fission yeast strains, i.e., the wild-type (SP223), $\Delta srk1$ (RA112), $\Delta chk1$ (SH4), $\Delta chk1/\Delta cds1$ (SH5), and $\Delta cds1$ (SH6) strains, were transformed with either pREP41x-*gfp-cdc25*/pYZ2N-*vpr* or pREP41x-*gfp-cdc25*, respectively. Cells were grown for 20 h in minimal EMM selective liquid medium in the absence of thiamine to induce gene expression. Cell images were captured to document the cell morphology, and the localization of Cdc25-GFP was visualized by viewing the emission of green fluorescence with a Leica fluorescence microscope. Immunoblot analysis was performed to confirm the proper expression of *vpr* in every yeast strain. The *vpr*-repressing and *vpr*-expressing cells were collected and lysed 20 h after gene induction. Equal amounts of proteins (25 μ g) were loaded onto SDS-PAGE gels, and Vpr was visualized with anti-Vpr (dilution, 1/500). The same levels of Vpr proteins were expressed in each of these samples (data not shown). (B) Deletion of the *srk1* gene has no obvious effect on the localization of Cdc25 under normal growth conditions. The *srk1*⁺ and $\Delta srk1$ yeast strains were transformed with pREP41x-*GFP*, pREP41x-*GFP-cdc25*, or pREP41x-*GFP-cdc25(9A)* and grown and assayed as described for panel A. Bar, 10 μ m.

respectively), coexpression of these two genes did not further elongate cells as expected from an additive effect. In fact, no significant difference ($n = 3$, $p > 0.1$) was measurable between wild-type cells expressing *srk1* alone (38.9 ± 4.4 μ m) and cells expressing both *srk1* and *vpr* (36.2 ± 1.7 μ m). Similarly, coexpression of the *vpr* gene with *srk1(K153A)* did not display any additive effect either, since this yielded a cell length of 18.9 ± 0.9 μ m, which is essentially the same ($n = 3$, $p > 0.01$) as for cells that only express *srk1(K153A)* (18.0 ± 0.5 μ m). Since the cell length measurement showed no additive effect, Vpr is most likely inducing cell cycle G_2 arrest by promoting the nuclear export of Cdc25 at least in part through Srk1.

To further test the genetic relationship between Vpr and Srk1 or whether Srk1 is required for Vpr-induced cell cycle G_2 arrest, a *vpr*-expressing fission yeast $\Delta srk1$ strain (SH2) was generated to measure Vpr-induced cell elongation in the absence of Srk1. Compared to the effect of Vpr on the wild-type (*srk1*⁺) cells, a significant reduction of Vpr-induced cell elongation was observed in the $\Delta srk1$ cells (Fig. 5B). As expected from the Vpr effect, an average cell length of 17.1 ± 0.1 μ m (Fig. 5B, top row) was observed in the *srk1*⁺ cells (4, 13), which is significantly longer ($n = 3$, $p \ll 0.001$) than for $\Delta srk1$ cells (12.2 ± 1.4 μ m) (Fig. 5B, bottom row). Thus, *srk1* gene deletion appeared to block Vpr-induced cell elongation. However, continued monitoring of the cell length in $\Delta srk1$ cells showed that the suppressive effect of the *srk1* deletion on Vpr-induced

cell elongation is temporary since the cell length increased to a level similar to that of wild-type cells (data not shown). Therefore, Srk1 is likely to be partially responsible for Vpr-induced cell cycle G_2 arrest.

Immunoprecipitation was also carried out to test potential Vpr-Srk1 interactions in vivo. HA-tagged Srk1, Srk1(K153A), or L3 was coproduced with Vpr in the RE007 strain (4). The L3 protein, which is an irrelevant ribosomal protein, was used as a negative control for detecting the specificity of the immunoprecipitation assay. HA-tagged Srk1 or L3 was first pulled down by the anti-HA antibody, and the presence of Vpr was detected by using anti-Vpr serum. As shown in Fig. 5C, lanes 6 and 9, Vpr was coimmunoprecipitated with both Srk1 and Srk1(K153A). However, no Vpr was detected in the immunoprecipitated L3 extracts (Fig. 5C, lane 12), suggesting that Vpr specifically interacts with Srk1. We noted, however, that Srk1(K153A) also binds Vpr with slightly less intensity. Since our data (Fig. 5A) suggested that the Srk1(K153A) mutant may have a residual kinase activity, this binding likely represents the residual kinase activity of Srk1. Taken together, these results indicate that Vpr induces cell cycle G_2 arrest in part through a direct association with Srk1.

MK2, a human homologue of Srk1 kinase, is required for Vpr-induced cell cycle G_2 arrest in mammalian cells. MK2 is a mammalian homologue of Srk1 (1, 55). Importantly, both Srk1 and MK2 directly phosphorylate and inhibit Cdc25 (35, 38). To

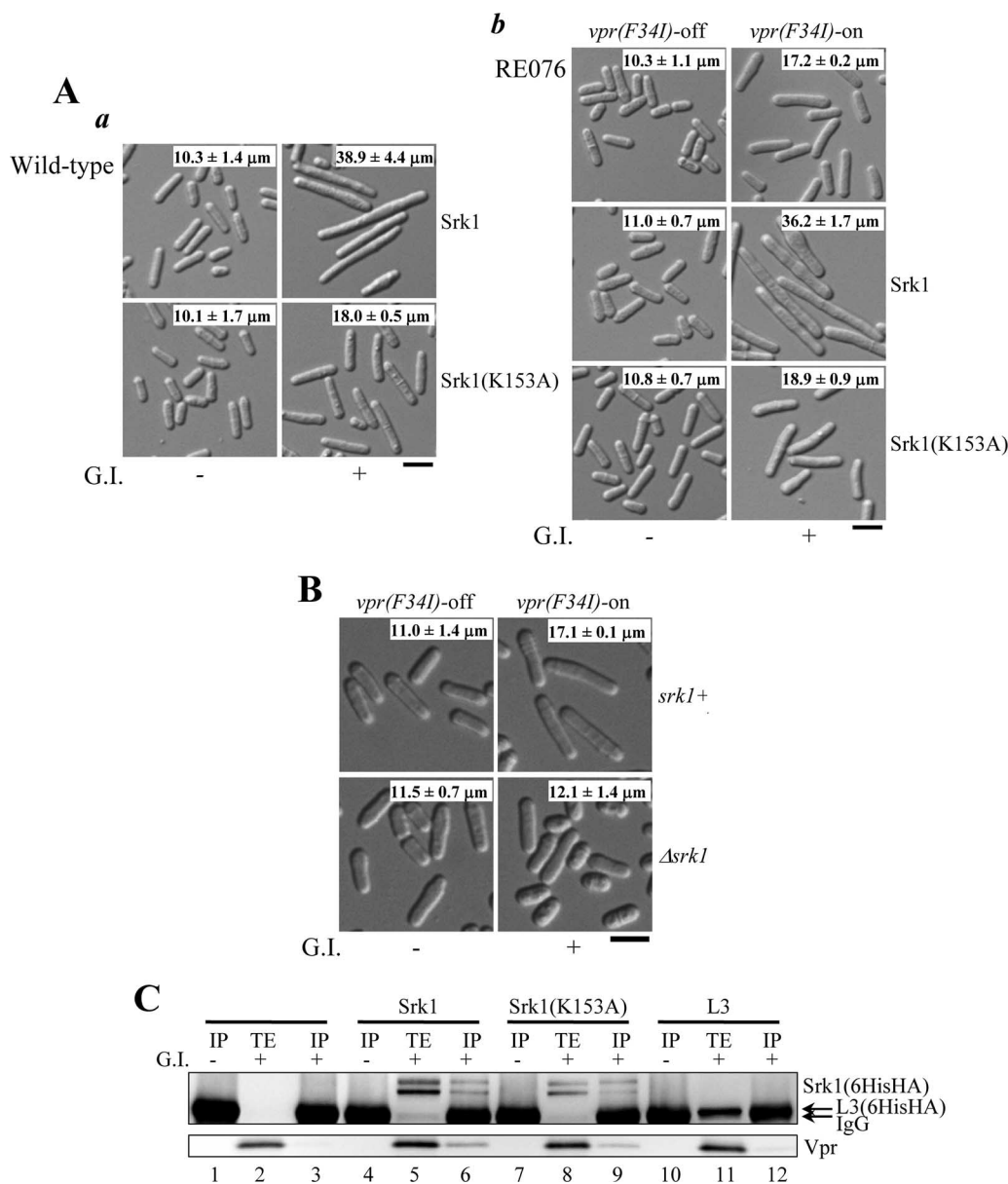
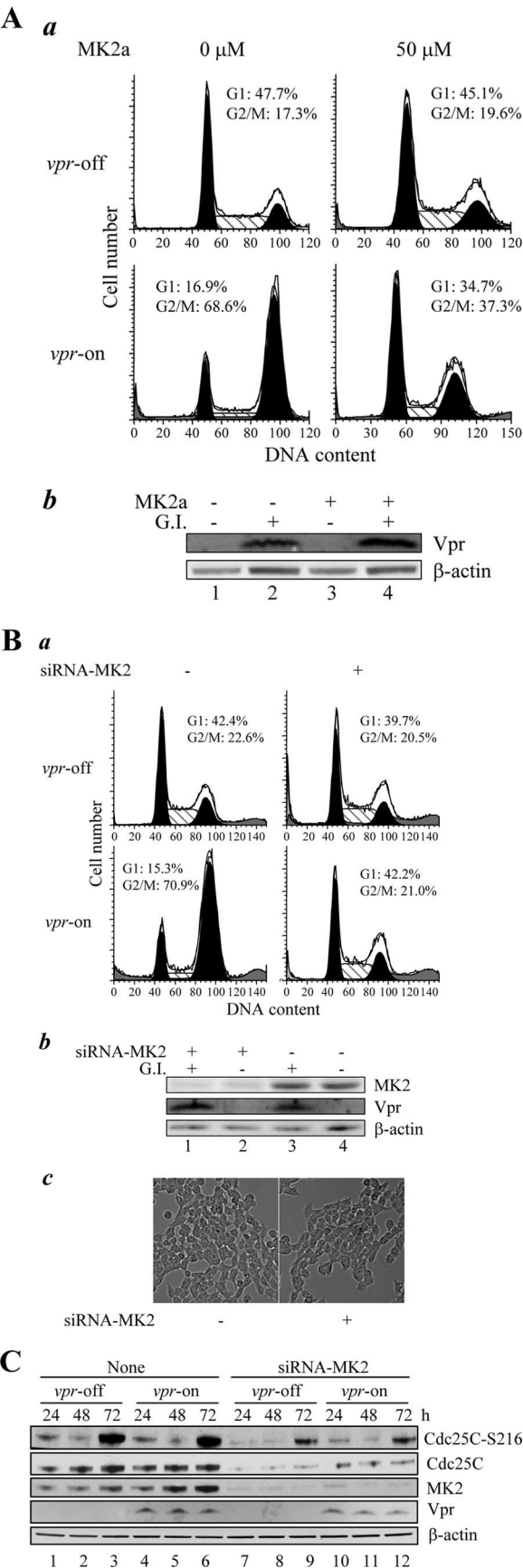


FIG. 5. Vpr induces cell cycle G₂ arrest through direct interaction with Srk1. (A) Coexpression of *vpr* and *srk1* showed no additive effect on Vpr-induced cell elongation. (a) Overexpression of *srk1* induces cell cycle G₂ arrest with cell elongation. The wild-type (SP223) yeast strain was transformed with pREP1-*srk1*(6HisHA) or pREP1-*srk1*(K153A)(6HisHA) and grown for 27 h in the minimal selective EMM liquid culture either in the presence or in the absence of thiamine to induce gene expression. The average cell length and standard deviation was measured on at least 100 cells by using the OpenLab software and based on three independent experiments. (b) Coexpression of *vpr* and *srk1* showed no additive effect on Vpr-induced cell elongation. The RE076 fission yeast strain, which carries a single integrated copy of F34Ivpr gene (13), was transformed with pREP1-*srk1*(6HisHA) or pREP1-*srk1*(K153A)(6HisHA) and grown and assayed as described for panel Aa. Immunoblot analysis was performed to confirm the expression of *srk1*(6HisHA), *srk1*(K153A)(6HisHA), and *vpr* in the SP223 and RE076 strains transformed with pREP1-*srk1*(6HisHA) or pREP1-*srk1*(K153A)(6HisHA). The same levels of Vpr or Srk1 proteins were expressed in each of these samples (data not shown). (B) Deletion of the *srk1* gene abolished Vpr-induced cell elongation. The *srk1*⁺ (RE076) and Δ *srk1* (SH2) fission yeast strains were grown for 20 h in the minimal selective EMM liquid culture in the presence [*vpr*(F34I)-off] or in the absence [*vpr*(F34I)-on] of thiamine to induce *vpr* gene expression. Cell length was measured as described for Fig. 1A. Immunoblot analysis was performed to confirm the expression of *vpr* in the *srk1*⁺ and Δ *srk1* strains. The *vpr*-repressing and *vpr*-expressing cells were collected and lysed after 20 h of gene induction. Equal amounts of proteins (25 μ g) were loaded onto SDS-PAGE gels, and the Vpr was visualized by using anti-Vpr (dilution, 1/500). The same levels of Vpr proteins were expressed in each of these samples (data not shown). (C) Vpr directly interacts with Srk1, as indicated by immunoprecipitation. The RE007 yeast strain was transformed with pREP1-*srk1*(6HisHA), pREP1-*srk1*(K153A)(6HisHA), or pREP1-L3(6HisHA) and was grown in the selective liquid culture for 24 h in the presence or in the absence of thiamine to induce gene expression. Srk1 was then immunoprecipitated with anti-HA antibody from cell extracts. The protein L3 was used here as a nonspecific protein control during the immunoprecipitation experiment. G.I., gene induction; IP, immunoprecipitation; TE, total extract. Bar, 10 μ m.



address the question of whether MK2, like *Srk1*, is involved in *Vpr*-induced cell cycle G₂ arrest in mammalian cells, a potent and specific inhibitor (MK2a) of the MK2 kinase was used to test its effect on *Vpr*-induced G₂ arrest. MK2a selectively inhibits p38 α -mediated MK2 phosphorylation (10, 37). A human DL-3 cell line, which was derived from an HEK293 cell carrying a stable and muristerone A-inducible *vpr* gene (34, 67), was used to test the MK2a effect. The expression of *vpr* was induced by the addition of muristerone A at a final concentration of 1 μ M. At 24 h prior to *vpr* gene induction, the MK2-specific inhibitor (MK2a) was added at a final concentration of 50 μ M. The cells were collected 72 h after gene induction for flow cytometry analysis. As we described previously (4, 33), the induction of *vpr* gene expression in the DL-3 cells resulted in a significant accumulation (68.6%) of the G₂/M cells, whereas the DL-3 cells without *vpr* gene induction had a normal cell cycle profile with only 17.3% of its cell population at the G₂/M phase (Fig. 6Ba). In contrast, no significant G₂ accumulation was observed in the *vpr*-expressing cells when they were treated with MK2a. The percentage of cells at the G₂/M phase decreased from 68.6 to 37.3% between *vpr*-repressing cells in the absence or presence of MK2a (Fig. 6Aa). Compared to the same MK2a-treated cells without *Vpr*, no significant difference (17.3% versus 19.6%) was seen between *vpr*-repressing cells in the absence or presence of MK2a (Fig. 6Ba), indicating that MK2a treatment has no obvious effect on the cell cycle profile. Therefore, these results suggest that MK2 kinase activity, like

FIG. 6. MK2, a human homologue of *Srk1*, is required for *Vpr*-induced cell cycle G₂ arrest in mammalian cells. (A) Treatment of *vpr*-expressing HEK293 cells with the MK2 inhibitor (MK2a) abolished *Vpr*-induced G₂ arrest. (a) Cell cycle profile analysis by flow cytometry. The DL-3 cells, which are derivatives of HEK293 carrying a stable and muristerone A-inducible *vpr* gene (34, 67), were grown in Dulbecco modified Eagle medium, and the expression of *vpr* was induced by the addition of muristerone A at a final concentration of 1 μ M. At 24 h prior to *vpr* gene induction, the MK2-specific inhibitor (MK2a) was added at a final concentration of 50 μ M. The cells were collected 72 h after gene induction for flow cytometry analysis. (b) Immunoblotting was performed to confirm the expression of *vpr* in DL-3 cells. The *vpr*-repressing and *vpr*-expressing cells were prepared as described for the flow cytometric analysis. Cells were collected and lysed 72 h after gene induction. Equal amounts of proteins (50 μ g) were loaded onto SDS-PAGE gels, and the *Vpr* was visualized by using anti-*Vpr* (dilution, 1/300). (B) Depletion of MK2 gene expression by siRNA abrogated *Vpr*-induced G₂ arrest. (a) The DL-3 cells were prepared as described for panel A. At 24 h prior to *vpr* gene induction, siRNA-MK2 was transfected into the cells. The cells were collected 72 h after gene induction for flow cytometry analysis. Reduction of the MK2 protein production was confirmed by the immunoblot analysis shown in (shown in panel Bb). (c) Images of HEK293 cell morphology with (+) or without (–) the knockdown of MK2 were captured by Leica microscopy with cells collected 72 h after the addition of siRNA-MK2. (C) Depletion of MK2 reduces Cdc25C-Ser216 phosphorylation and partially restores the Cdc25C protein level in the presence of *Vpr*. The DL-3 cells described for panel Bc with or without the knockdown of MK2 were collected at the indicated times. The proteins extracted were subjected to the Western blot analysis. Phosphorylation of Cdc25C at the Ser216 residue was detected by using monoclonal anti-Cdc25C antibody (Cell Signaling). Total protein levels of Cdc25C and MK2 were detected by using a monoclonal antibody (UpState) and polyclonal anti-MK2 antibody (Cell Signaling), respectively.

that of *Srk1* kinase, is at least in part required for Vpr-induced cell cycle G_2 arrest.

Even though the MK2a is a specific inhibitor of MK2 (10, 37), a chemical inhibitor could have other uncovered effects that may have obscured the observed results. To verify the specific involvement of MK2 in Vpr-induced G_2 arrest, we took advantage of the siRNA technology to specifically downregulate the production of MK2 protein. As shown in Fig. 6Bb, greatly reduced protein levels of MK2 were detected when the DL-3 cells were transfected with a commercial siRNA that was designed and validated to specifically silence the expression of the *MK2* gene (Ambion). To test the depleting effect of MK2 on Vpr-induced G_2 arrest, the specific anti-MK2 siRNA was transfected into the DL-3 cells 24 h before the induction of the *vpr* gene expression. The *vpr*-expressing cells were collected 72 h after gene induction for flow cytometry analysis (Fig. 6Ba). As shown in Fig. 6Ba, the cells displayed normal cell cycle profiles regardless of the siRNA treatment, indicating that the siRNA had no significant effect on the cell cycle. Consistent with the idea that Vpr induces cell cycle G_2 arrest in DL-3 cells (4, 33), the *vpr*-expressing cells had a marked accumulation of G_2 cells. The percentage of G_2 cells increased from 22.6% without Vpr to 70.9% with Vpr (Fig. 6Ba). However, the number of G_2 cells was markedly reduced in cells in which MK2 was depleted by siRNA (21.0% versus 70.9%). To ensure that the observed suppression of Vpr-induced G_2 arrest in the *MK2* knockdown cells was not due to a potential side effect on cell proliferation or viability, three additional experiments were carried out to compare the doubling times, cell morphology, and cell death of the HEK293 cells with or without knockdown of MK2. As shown in Fig. 6Bc, there were few or no obvious changes of cell morphology between the MK2 knockdown cells and the control cells. Measurement of cell growth over a period of 96 h showed that the average doubling time for the control cells was 24.7 h, whereas a significant reduction in growth was seen in the MK2 knockdown cells, with an average doubling time of 63.7 h. However, very little cell death was detected in either cell type when trypan blue staining was used to detect possible dead cells. An average of $3.89\% \pm 1.95\%$ dead cells was observed in the MK2 knockdown cells, with $1.64 \pm 0.88\%$ dying cells seen in the control cells.

Since *Srk1*/MK2 recognize the same phosphorylation sites on Cdc25/Cdc25C as on *Chk1*/*Chk2* (35, 38), we tested the potential effect of *MK2* depletion on the total Cdc25C protein level and phosphorylation on Ser216. DL-3 cells with or without *vpr* gene expression were collected 24, 48, or 72 h after gene induction. Somewhat similar to our observations in fission yeast, Vpr appears to affect the total protein levels of Cdc25C by conferring small levels (Fig. 6C, row 2, lanes 4 to 6) to moderate levels (data not shown) of reduction. Interestingly, depletion of *MK2* resulted in a significant reduction in the Cdc25C (Fig. 6C, lanes 7 to 12). It is clearly noticeable, however, that small increases in the Cdc25C protein levels were consistently observed in the *vpr*-expressing cells compared to the *vpr*-repressing cells (Fig. 6C, lanes 10 to 12 versus lanes 7 to 9). These data suggest that MK2 may normally stabilize Cdc25C protein but that, in the presence of Vpr, Vpr mediates its effect through MK2 and results in a small reduction in Cdc25C observed in the wild-type cells.

Despite the small differences in Cdc25C protein levels found between the *vpr*-repressing and *vpr*-expressing cells, an increasing phosphorylation of Cdc25C-Ser216 was observed over time, with the highest phosphorylation levels observed at 72 h after gene induction (Fig. 6C, row 1, lane 6), suggesting that Vpr promotes the phosphorylation of Cdc25C-Ser216. Noticeably, however, a very strong phosphorylation of Cdc25C was also observed in *vpr*-suppressing cells (Fig. 6C, lane 3). Since Cdc25C-Ser216 phosphorylation does not accumulate through multiple cell cycles, this increase was presumably due to the low-level expression of *vpr* that is commonly found in many of the inducible gene systems (46, 59). Significantly, the depletion of *MK2* strongly reduced the phosphorylation levels of Cdc25C-Ser216 in both *vpr*-repressing and *vpr*-expressing cells, indicating that MK2 indeed promotes at least in part the phosphorylation of Cdc25C-Ser216.

DISCUSSION

We demonstrated here that Vpr-induced cell cycle G_2 arrest is mediated at least in part through modulation of the *Srk1*/MK2 kinase, which exerts inhibitory effects on the Cdc25 phosphatase (Fig. 1B and C). Similar to the cellular checkpoint responses to DNA damage or inhibition of DNA replication, Vpr expels Cdc25 from the nucleus to the cytoplasm, where it can no longer access Cdc2/Cdk1; thus, Cdc25 is no longer able to promote cellular entry to mitosis. The similarity between the effect of Vpr and the DNA checkpoints upon nuclear export of Cdc25 is further extended to the requirement for Cdc25 phosphorylation (Fig. 1B) and the dependency of Rad24 (Fig. 2A) on an active Crm1-mediated nuclear-cytoplasmic shuttling (Fig. 2B). Unlike the mitotic DNA checkpoints, however, genetic mutants that are defective in the early (*rad3*) or late (*chk1*, *cds1* and *chk1/cds1*) mitotic DNA checkpoint pathway are not directly involved in the nuclear exclusion of Cdc25 induced by Vpr (Fig. 3B and 4A), suggesting that Vpr may use an alternative signaling pathway to drive out Cdc25 in *S. pombe*. Moreover, since Cdc25 is typically phosphorylated either by *Chk1* or *Cds1* during cellular mitotic checkpoints (6, 60), our data suggest that *Srk1*, a different kinase that phosphorylates Cdc25 in a similar manner to *Chk1* or *Cds1* during the normal cell cycle (35), is required for the nuclear export of Cdc25 (Fig. 4A). A requirement for the *Srk1*-like kinase in Vpr-induced cell cycle G_2 arrest in mammalian cells was further confirmed by the siRNA-mediated depletion of the *MK2* gene, a mammalian homologue of *Srk1* that decreases the G_2 arrest (Fig. 6A). Moreover, inhibiting the MK2 kinase activity with a specific inhibitor MK2a exhibits a similar level of reduction of the G_2 arrest (Fig. 6B), supporting the idea that *Srk1*/MK2 kinase is required at least in part for the induction of cell cycle G_2 arrest by Vpr.

Cell cycle G_2 arrest induced by Vpr is attributed to the inhibitory hyperphosphorylation of Cdc2/Cdk1 both in fission yeast and in mammalian cells (12, 22). The phosphorylation status of Cdc2/Cdk1 is normally regulated by the Wee1 kinase and the Cdc25 phosphatase during the normal cell cycle. In the situation of the mitotic DNA checkpoint controls, either the Wee1 kinase could be upregulated or the Cdc25 phosphatase could be downregulated. Consequently, the Cdc2/Cdk1 becomes inactive due to hyperphosphorylation that leads to cell

cycle G₂/M arrest. In the case of Vpr-induced G₂ arrest, both activation of the Wee1 kinase and inhibition of the Cdc25 phosphatase have been reported (3, 13, 18, 25, 41, 42, 58). However, the molecular mechanism underlying the inhibitory effect of Vpr on Cdc25 is not fully understood. The requirement of Cdc25 in Vpr-induced G₂ arrest was initially shown in fission yeast (13), in which double mutations of *cdc25* and *wee1* synergistically reduced the G₂ induction by Vpr, and in vitro Cdc25 activity analysis further showed that Vpr inhibits Cdc25C activity and further showed that the inhibitory effect of Vpr on Cdc25 is conferred by direct Vpr-Cdc25C interaction (18). However, the results of that study raised a question as to whether the Cdc25C phosphatase activity or the binding of Vpr to Cdc25C is necessary for Vpr-induced G₂ arrest since the expression of both a catalytically active mutant of Cdc25C that has reduced binding to Vpr and a catalytically inactive mutant of Cdc25C that retains binding to Vpr was able to largely overcome the Vpr-mediated G₂ arrest (18). Other studies in fission yeast and mammalian cells suggested that nuclear exclusion of Cdc25, a typical cellular mitotic DNA checkpoint response (36), may also play a role in Vpr-induced G₂ arrest (13, 25). In fission yeast, the overexpression of *rad25*, a sibling gene of *rad24* and a human homologue of 14-3-3, markedly enhanced Vpr-induced G₂ arrest (13). Both Rad25 and Rad24, like mammalian 14-3-3, inhibit Cdc25 in fission yeast by exporting phosphorylated Cdc25 from the nucleus to the cytoplasm (36, 49). This finding was further verified in mammalian cells, in which 14-3-3 overexpression synergized with Vpr in the arrest of the cell cycle (25). Moreover, Vpr interacts directly with both 14-3-3 and Cdc25C (25). Consistent with the role of 14-3-3 in the nuclear exporting of Cdc25, *vpr* expression indeed shifted the localization of the Cdc25C S216A mutant to the cytoplasm. The Cdc25C S216A mutant was used presumably because the wild-type Cdc25C predominantly resides in the cytoplasm of the normal proliferating human cells, which are mostly in the G₁ phase of the cell cycle (9, 11, 20, 36). The S216A mutation, which partially interrupts the binding of 14-3-3 to Cdc25C (8), moderately shifted Cdc25C to the nucleus, thus allowing testing of the effect of Vpr on the nuclear export of Cdc25. Interestingly, however, the expression of *vpr* was able to push the Cdc25C S216A back to the cytoplasm (25), raising the question of whether the 14-3-3 is indeed needed for nuclear transport of Cdc25C or whether perhaps additional phosphorylation sites are also involved in the binding of 14-3-3 to Cdc25C. Resolving this issue is difficult in mammalian cells since there are three Cdc25 homologues. To answer this question, we turn to the fission yeast model system, in which only one Cdc25 phosphatase is present. The results our studies showed that, as in mammalian cells, the expression of *vpr* does promote the nuclear export of Cdc25. In addition, this nuclear exporting process requires Rad24, since the deletion of *rad24* largely blocked the nuclear export of Cdc25 (Fig. 2A). The requirement for Rad24 and the possible binding of Rad24 to Cdc25 for the nuclear-cytoplasmic shuttling was further strengthened by our finding shown that amino acid substitution of all nine serine/threonine phosphorylation sites of Cdc25 with alanine [Cdc25(9A)] not only interrupted the Rad24/14-3-3 binding to Cdc25 (39, 40, 57) but also completely abolished the cytoplasmic shift of Cdc25 in-

duced by Vpr (Fig. 1Ba, bottom row). Given the fact that Cdc25 dephosphorylates Cdc2/Cdk1 in the nucleus to promote mitosis, it seems likely that the most efficient way to prevent phosphorylation of Cdc2/Cdk1 by Cdc25 is to keep it away from the nucleus. Therefore, we propose that Vpr promotes an active nuclear transport of Cdc25 via a Crm1-mediated mechanism that leads to the sequestration of this phosphatase into the cytoplasm and away from its nuclear substrate of Cdc2. Vpr might modulate Cdc25 by changing the binding specificity to Rad24/14-3-3 through the phosphorylation status of Cdc25.

Until now, it was unknown which kinase is responsible for the phosphorylation of Cdc25 induced by Vpr. In cellular responses to DNA damage or inhibition of DNA replication, Cdc25 is typically phosphorylated either by Chk1 or by Chk2 (27, 36, 49, 54, 61). However, our data showed here that neither Chk1 nor Cds1 is required for the Vpr-mediated nuclear export of Cdc25 (Fig. 4A). These findings are consistent with previous genetic studies in fission yeast showing that the deletion of *chk1* and/or *cds1* does not suppress Vpr-induced cell cycle G₂ arrest (13, 41, 42). In the present study, we demonstrated that a different Srk1 kinase that regulates the G₂/M transition through inhibitory phosphorylation of Cdc25 during normal cell cycle (35) is specifically involved in the nuclear export of Cdc25 induced by Vpr. The specific requirement of Srk1 by Vpr is demonstrated by the observation that the deletion of the *srk1* gene almost completely prevented the nuclear transport of Cdc25 (Fig. 4A, bottom row). This finding is in sharp contrast to the deletions of *chk1* and/or *cds1*. Neither deletion, when tested individually or combined, was able to reduce the shift of Cdc25 to the cytoplasm (Fig. 4A). Our data further suggested that both Vpr and Srk1 work in concert to inhibit Cdc25. The fact that no additive effect of Vpr/Srk1-induced cell elongation was observed when *vpr* and *srk1* were coexpressed suggests they both exert their inhibitory effect on Cdc25 through the same pathway. That the deletion of *srk1* blocks the nuclear transport of Cdc25 (Fig. 4A) and Vpr-induced cell elongation (Fig. 5B) further indicates that Vpr is most likely located upstream of Srk1 in the signaling pathway leading to cell cycle G₂ arrest. It was noticed, however, that the blocking of Vpr-induced cell elongation by Δ *srk1* is only temporary since these *vpr*-expressing cells ultimately became elongated (data not shown). This phenomenon suggested two possibilities. First, there might be another kinase that can phosphorylate Cdc25 in the absence of Srk1. This possibility is unlikely because the deletion of *srk1* blocks the nuclear transport of Cdc25 over the entire testing period (Fig. 4A). Alternatively, the observed effect may have been caused by upregulation of the Wee1 kinase induced by Vpr. In this case, upregulated Wee1 can cause hyperphosphorylation of Cdc2/Cdk1 even if the Cdc25 phosphatase is at its normal physiological level. This possibility is certainly supported by early studies from both fission yeast and mammalian cells showing that increased levels of the Wee1 kinase are necessary for Vpr-induced G₂ arrest (42, 58). With regard to the relationship of Vpr and Srk1, the results of the coimmunoprecipitation experiment further showed that the inhibition of Cdc25 by Vpr is modulated through the Srk1 kinase via direct protein-protein interaction (Fig. 5C). It was noted that Srk1(K153A), a catalytically inactive mutant (35), also binds to Vpr. We assume that this is because the residual kinase activity left in this

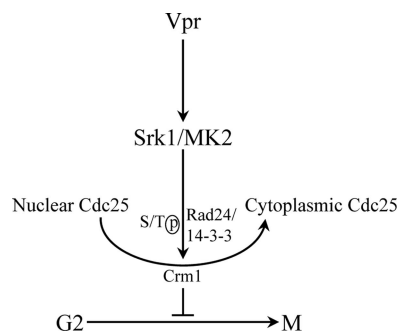


FIG. 7. Putative model for Srk1/MK2-mediated nuclear-cytoplasmic shuttling of Cdc25 during cell cycle G_2 arrest induced by Vpr.

mutant as overexpression of the *srk1*(K153A) gene also induced cell elongation to a level that is similar to that for Vpr but much less than that for the wild-type Srk1 (Fig. 5A).

Finding a fission yeast kinase, such as Srk1, other than Chk1 or Cds1, involved in the inhibitory phosphorylation of Cdc25 induced by Vpr is physiologically relevant to the Vpr effect in mammalian cells because the inhibition of the MK2 kinase, a mammalian homologue of Srk1, showed a similar strong reduction of Vpr-induced G_2 arrest in mammalian cells. However, whether the mammalian Chk1 or Chk2 is also involved in the inhibitory phosphorylation of Cdc25 is unknown at present. Since ATM activates Chk1 and ATM is not specifically involved in Vpr-induced G_2 arrest (3), it is less likely that Chk2 participates in the inhibition of Cdc25-induced Vpr. Chk1, however, has been shown to be specifically required for Vpr-induced G_2 arrest (28, 33, 53). Whether Chk1 is also activated by Vpr to phosphorylate Cdc25 and to promote the nuclear export of Cdc25 has yet to be tested. Interestingly, unlike Srk1, the mammalian MK2 has recently been implicated in the DNA damage checkpoint, since MK2-deficient cells display an almost complete loss of the UV-induced G_2 checkpoint (38). Since the DNA damage checkpoint is less likely involved in Vpr-induced G_2 arrest (3), it is unclear at the moment how MK2 fits into the checkpoint scheme of the Vpr effect. Alternatively, the finding that MK2 participates in the DNA damage checkpoint could represent another example of the redundancy of mammalian proteins in executing a specific cellular function (62, 63). On the other hand, since Srk1 has not been implicated in DNA checkpoints, our findings may emphasize the unique aspect of the Vpr effect on the host cell cycle regulation compared to the classic mitotic DNA checkpoints.

In summary, we report here that Vpr induces cell cycle G_2 arrest in part by the inhibitory phosphorylation and nuclear export of Cdc25, which is modulated through the Srk1/MK2 kinase. Specifically, we propose that Vpr activates the Srk1/MK2 kinase by direct protein-protein interaction (Fig. 7). The activated Srk1/MK2 phosphorylates Cdc25 at the serine/threonine sites, which promotes the binding of Rad24/14-3-3 to Cdc25. The association of Rad24/14-3-3 with Cdc25 leads to the export of Cdc25 from the nucleus to the cytoplasm through an active nuclear-cytoplasmic shuttling involving Crm1. Consequently, the nuclear exclusion of Cdc25 prevents the dephosphorylation of Cdc2/Cdk1 by Cdc25 that results in the hyperphosphorylation of Cdc2/Cdk1, leading to the cell cycle G_2 arrest.

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